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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Katia Georgopoulos et al.
Serial No. : 09/259,389
Filed : February 26, 1999
Title : HELIOS GENE

Art Unit : 1632
Examiner : J. Woitach

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Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. §1.132 OF DR. KATIA GEORGOPOULOS

I, Katia Georgopoulos, a citizen of the United States of America, residing in Lexington, Massachusetts, hereby declare as follows:

1. I am currently Associate Professor of Dermatology (Anatomy and Cellular Biology) at Massachusetts General Hospital.
2. I am a co-inventor of the invention claimed in the above-identified patent application, and I have read and understand the contents of the present patent application.
3. I have also been advised and understand that the Examiner has rejected claims 1, 3, 5, 10, 11, 13 and 18-21 of the above-referenced application which are directed to a nucleic acid that encodes an amino acid sequence that is at least 80% identical to the amino acid sequence of human Helios polypeptide (SEQ ID NO:6). I further have been advised and understand that this rejection, in part, is based on the Examiner's assertion that it is not clear to

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what extent one can alter the sequence of SEQ ID NO:6 and still maintain any Helios biological activity.

4. It would not require undue experimentation for one skilled in the art to determine what sections of Helios to alter to make Helios proteins having (or not having) a particular activity. As discussed in detail below, the structure and function of Helios is well documented in the specification. In addition, the structure and function of a highly related protein, Ikaros, is well documented in the art. Mutational and isoform analyses have confirmed the functions of the predicted domains of Ikaros. Therefore, given the degree of similarity between Ikaros and Helios, one skilled in the art would expect that similar mutations would have similar effects in Ikaros and Helios.

5. As is discussed in the specification, the Helios polypeptide is part of the Ikaros family of transcription factors. Helios and Ikaros are characterized, in part, by having: four N-terminal zinc finger domains which make up the DNA binding domain, two C-terminal zinc fingers that mediate protein dimerization, and a transcriptional activation domain (see, for example, Figure 1 of the application and accompanying legend). As discussed in the application at pages 61-62, the proteins Helios and Ikaros share 76% sequence similarity overall; 93% sequence identity from the first thorough the fourth zinc fingers; 86% sequence identity in the protein dimerization domain, which includes the C-terminal zinc fingers; and 68% sequence similarity in the transcriptional activation domain. The defining characteristics of the Helios protein are extensively described in the present application, as described below.

6. The general structure of Helios is discussed throughout the specification. For example, Figure 1 shows the structure and placement of the various domains and zinc finger motifs of the Helios protein. The accompanying legend at page 35, lines 19-24, provides that "the four N-terminal zinc fingers (ZF1-4) comprising the DNA binding domain, the C-terminal zinc fingers (ZF5-6) that mediate protein dimerization and the conserved transcriptional activation domain (TAD) are outlined" in Figure 1.

7. A great deal of information is also provided in the specification with regard to Helios' DNA binding and transcriptional activation activities. See page 62, lines 17-19, where the specification provides that "strong conservation of the N-terminal zinc finger motifs of Hel-1 and Hel-2 with Ikaros isoforms Ik-1 and Ik-2 predicts that they will display similar affinities and DNA binding specificities." The affinities and DNA binding specificities of Ikaros isoforms Ik-1 and Ik-2 were known at the time of filing and are disclosed in Molnar and Georgopoulos (1994) Mol Cell Biol. 14:8292-303. The application further provides as follows.

Given the near identity in the DNA binding domain between Helios and Ikaros, we tested the ability of Helios to activate transcription from Ikaros binding sites. The expression of a reporter gene under the control of four high affinity Ikaros binding sites (IkBS2) was tested in the presence of Helios or Ikaros in NIH3T3 cells. Both proteins were shown to increase expression of the reporter gene over background levels (Figure 3). A five fold increase was detected in the presence of Helios while a 7.8 fold increase was detected in the presence of Ikaros. This transcriptional activation mediated by Helios requires the Ikaros consensus binding sites. These results confirm the functional conservation of both the DNA binding and transcriptional activation domains. (specification at page 72, lines 5-19, emphasis added).

8. Thus, the application not only teaches the location of the DNA binding and transcriptional activation domains within the Helios protein, but also teaches the use of a reporter gene assay that one can use to determine if a protein has the DNA binding and transactivation activity (namely, the reporter gene assay described in the above quoted passage).

9. In addition, a great deal of information is provided in the specification with regard to Helios' dimerization activity. For example, the specification discloses that Helios can form homodimers with itself or heterodimers with Ikaros or Aiolos. The paragraph bridging pages 69-70 of the specification provides as follows.

As mentioned above, to determine whether Helios isoforms Hel-1 and Hel-2 can form dimers with self, as well as with Ikaros and Aiolos, these factors were transiently expressed in 293 T cells in pairwise combinations. One protein in each expressed pair was epitope tagged (FLAG). After two days, cell lysates were prepared and Western blot analysis confirmed protein expression using antibodies specific for each of the Helios, Ikaros and Aiolos proteins. An

antibody to the epitope tag (anti-FLAG) was used to immunoprecipitate complexes from 293T cell lysates, and precipitated complexes were analyzed for protein interactions using Ikaros or Helios specific antibodies. The anti FLAG antibody co-precipitates both FLAG-Hel-1 and Hel-2, demonstrating that the two isoforms can dimerize. A similar strategy was used to study Helios, Ikaros and Aiolos interactions. FLAG-Hel-1 or FLAG-Hel-2 were co-expressed with Ik-1 . The anti-FLAG antibody brought down IK-1 in an immunoprecipitated complex in both cases. To control for the specificity of the Helios/Ikaros protein interactions, the IkM1 (Ik-1 mutant) was also used in these assays. IkM1 encodes two point mutations in the C-terminal zinc fingers of Ikaros that disrupt the ability to dimerize. In contrast to Ik-1, this dimerization deficient form of Ikaros was unable to interact with either Helios isoform. Finally, cells were co-transfected with FLAG-Aiolos and either Hel-1 or Hel-2 to show that each Helios isoform can form heterodimers with Aiolos. These studies show that the C-terminal zinc fingers in Helios, Ikaros and Aiolos are functionally conserved and mediate the stable interactions between these proteins which may be critical for hematopoiesis as well as lymphocyte differentiation and function.

Thus, the disclosure (a) teaches that Helios has homo-and hetero-dimerization activity, (b) provides information about what part of the Helios structure is responsible for that activity, namely the C-terminal zinc fingers, and (c) provides an immunoprecipitation assay using FLAG tagged proteins that can be used to determine if a protein has dimerization activity.

10. In addition, I have performed substantial mutational analysis of the Ikaros protein sequence. In brief, the four N-terminal zinc fingers which conform to the Kruppel consensus bind to DNA and are not involved in protein-protein interactions. In contrast the two C-terminal zinc fingers present in all of the Ikaros proteins do not engage in high affinity DNA binding but potentiate strong protein-protein interactions. The transcriptional activation domain mapped to an 81 amino acid region shared by all of the Ikaros isoforms located upstream of the C-terminal zinc finger dimerization motifs. This analysis, described in more detail below, shows that each of the identified domains function as predicted in the Ikaros polypeptide. Given the degree of structural similarity between Ikaros and Helios, the domains of the Helios protein can be predicted to function in a manner similar or analogous to the domains of Ikaros. In fact, I have not performed analogous experiments with Helios precisely because I believe that the results of the Ikaros experiments with regard to basic structure and function extend to Helios as well.

Therefore, an ordinary skilled artisan could predictably modify the Helios protein sequence to obtain a protein with or without a desired activity.

11. With regard to DNA binding, experiments with Ikaros showed that the N-terminal zinc finger region is involved in DNA binding. Four of the Ikaros isoforms (Ik-1, Ik-2, Ik-3 and Ik-4), which contains from two to four N-terminal zinc fingers, bind to sequences that share the 4 bp motif GGGGA. Nevertheless, their overall sequence specificities and affinities for DNA are distinct (Molnar and Georgopoulos, 1994). For example, three of the four N-terminal zinc fingers required for sequence-specific DNA binding. Two of the Ikaros isoforms (Ik-5 and Ik-6), which have one and no N-terminal zinc fingers, do not bind DNA. Accordingly, one of ordinary skill in the art could easily predict that deleting or mutating the 4 N-terminal zinc finger region in Helios would abolish its DNA binding activity and would not affect dimerization activity. One could also predict that mutating or deleting only some of the Zinc fingers in Helios would affect sequence specificities and affinities for DNA (as in Ikaros), while not completely abolishing DNA binding.

12. The transcriptional activation domain of Ikaros was delineated using the yeast one-hybrid system and GAL-4 transactivation assays in mammalian cells. Ikaros peptides and proteins were fused to a nuclear localization signal and to the LexA DNA binding domain in the pL202 vector. Their activities in transcription were assayed in the yeast strain EGY48 (See Sun et al. Figure 1A and 6A). The first 154 amino acids of exon 7 exhibited strong transactivation properties in both the β -galactosidase and the Leu2 assays (See Sun et al. Figures 1A and 6A, A2 domain). Further deletion analysis mapped an activation domain within the first 81 amino acids of exon 7 (See Sun et al. Figures 1A and 6A, A4 domain). This region is shared by all of the Ikaros isoforms and is located upstream of the zinc finger dimerization motifs. The 81 amino acid activation region was capable of stimulating expression of both the β -galactosidase and the Leu2 genes. Further dissection of this region revealed a stretch of acidic amino acids essential for this activity. The acidic amino acid sub domain could function alone as a weak activator, as indicated by its ability to stimulate growth in the Leu- assay but not color development in the β -galactosidase assay (See Sun et al. Figure 6A, A6). An adjacent stretch of residues containing

hydrophobic amino acids was required for maximal activity of the Ikaros activation region. Thus, one could predict that mutating or deleting the corresponding region in Helios would affect Helios' ability to stimulate transcription.

13. The presence of a protein interaction domain in Ikaros was investigated in the yeast two-hybrid system. Of the Ikaros protein domains tested in this assay, the 154 C-terminal amino acids of exon 7 interacted with self and with the full-length Ikaros isoforms in both prey and bait vector configurations (See Sun et al. Figure 1B, A3). 5' and 3' deletional analysis of this region delineated a minimal interaction domain of 51 C-terminal amino acids which contains two Kruppel-like zinc finger motifs (See Sun et al. Figure 1B, I2, F5 and F6). These zinc finger motifs are not involved in high affinity DNA interactions. Thus, interactions between the Ikaros proteins are mediated by two zinc finger motifs (F5 and F6) located within the most C-terminal 58 amino acids. The F5 and F6 Kruppel-like fingers essential for Ikaros protein interactions are present in all of the Ikaros isoforms and in Helios as well. Therefore, one of ordinary skill in the art could easily predict that, e.g., deleting or mutating this region in Helios would abolish its dimerization activity and would not affect DNA binding activity.

14. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Date

Katia Georgopoulos, Ph.D.